

## IMMUNOASSAY USING HAPTEN-ENZYME CONJUGATES

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### 1. Introduction

Recently, we described an "enzyme-immunoassay" for human chorionic gonadotrophin (HCG<sup>\*</sup>), using the enzyme horse radish peroxidase (HRP) as a label for the antigen instead of radioactive iodine, as used in radioimmunoassay [1]. Independently, Engvall et al. developed similar assay systems with alkaline phosphatase-labelled antigen [2, 3] or antibody [4].

We have now applied the enzyme-immunoassay principle to the determination of haptens<sup>\*\*</sup>. We used oestradiol and oestriol, coupled through succinyl bridges to HRP, and antibodies against these steroid hormones. The resulting assay systems are more or less analogous to the radioimmunoassays for steroid hormones [5] that have been developed after the initial demonstration that steroid hormones can act as haptens [6, 7].

### 2. Materials and methods

#### 2.1. Reagents

Oestradiol-17 $\beta$ -hemisuccinate and oestriol-16 $\alpha$ , 17 $\beta$ -dihemisuccinate were prepared by heating the oestrogens with excess succinic anhydride in pyridine, followed by hydrolysis of the 3-ester with dilute sulphuric acid and by repeated crystallisation from methanol. HRP, grade

IV, R.Z. 0.6, was purchased from Miles-Seravac; BSA, Cohn fraction V, from Armour; sec. butylchloroformate and tri-*n*-butylamine from Fluka; 5-aminosalicylic acid from EGA-Chemie. Various oestrogen sulphates and glucuronides were bought from Sigma. Lactalbumin was from De Meyerij, Veghel, The Netherlands. Dasp anti-rabbit (sheep antibodies against rabbit  $\gamma$ -globulin, covalently linked to cellulose) is an Organon product.

#### 2.2. Preparation of oestrogen-protein conjugates

E-BSA and E-HRP were prepared by a mixed anhydride reaction, using sec. butylchloroformate. E-BSA was prepared essentially according to Erlanger et al. [8], using a molar ratio E/BSA of 60. The number of E-residues per BSA molecule in the conjugates was estimated, both spectrophotometrically and by amino group determination with trinitrobenzene sulphonic acid [9], to be about 25 for E<sub>2</sub>-BSA and 32 for E<sub>3</sub>-BSA. E-HRP conjugates were prepared using E/HRP ratios ranging from 5 to 40 (see Results). After the reaction they were passed over a Sephadex G-50 column, dialyzed and lyophilized, before subjecting them to further purification procedures (see Results).

#### 2.3. Preparation of antisera against E-BSA

Rabbits received 3  $\times$  1.25 mg immunogen subcutaneously in complete Freund's adjuvant, with 14-day intervals, followed by an intravenous injection of 1.25 mg immunogen in physiological saline. The rabbits were bled one week after the booster injection. If necessary, the animals received a second series of injections.

\* Abbreviations used are: HCG: human chorionic gonadotrophin; HRP: horse radish peroxidase; E: oestrogen; E<sub>1</sub>: oestrone; E<sub>2</sub>: oestradiol; E<sub>3</sub>: oestriol; BSA: bovine serum albumin.

\*\* Dutch patent application 7016396, filed 10th November 1970.

#### 2.4. Enzyme-immunoassay procedures

The double antibody solid phase method was used, employing Dasp anti-rabbit (sheep antibodies against rabbit  $\gamma$ -globulin, covalently linked to cellulose) [10, 11]. Incubation times and buffer were the same as in the Dasp enzyme-immunoassay for HCG [1], except for the phosphate concentration of the buffer, which was increased to 0.1 M in order to avoid non-specific adsorption of E-HRP conjugates to the immunosorbent.

#### 2.5. Peroxidase assay

HRP-activity was determined with 5-aminosalicylic acid as oxygen-acceptor, as described earlier [1].

### 3. Results

E-HRP conjugates were prepared while using molar E/HRP ratios ranging from 5 to 40. Fig. 1 shows the relation between E/HRP ratio and "immune reactivity", defined as the percentage of the enzyme activity that can be bound by excess antibody against the corresponding steroid, for E<sub>2</sub>-HRP and E<sub>3</sub>-HRP. Since virtually no enzyme activity is lost during the coupling reaction, the yield of each conjugate before purification equals its immune reactivity.

An E<sub>2</sub>-HRP and an E<sub>3</sub>-HRP, both prepared with an E/HRP ratio of 20, were subjected to various purifi-

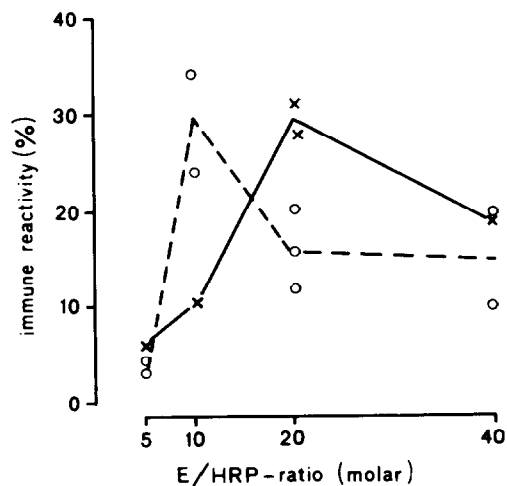


Fig. 1. Effect of E/HRP ratio (molar) during coupling on the immune reactivity of the unpurified E-HRP conjugates.

(x—x—x) E<sub>2</sub>-HRP; (o---o---o) E<sub>3</sub>-HRP.

Table 1  
Purification of E-HRP conjugates by various methods.

| Purification step                     | E <sub>2</sub> -HRP   |         | E <sub>3</sub> -HRP   |                  |
|---------------------------------------|-----------------------|---------|-----------------------|------------------|
|                                       | Immune reactivity (%) | Yield * | Immune reactivity (%) | Yield *          |
| Non-purified                          | 28                    | 28      | 20                    | 20               |
| Sephadex G-75                         | 48                    | 10      | 34                    | 14               |
| BioGel P-60                           | 41                    | 6       | 27                    | 8                |
| Density gradient ultra-centrifugation | 64                    | 5       | 95 **<br>56 **        | 0.2 **<br>0.8 ** |

\* Yield is expressed as:  
Immune reactivity product  $\times \frac{\text{enzyme activity product}}{\text{initial enzyme activity}}$

\*\* The E<sub>3</sub>-HRP ultracentrifuge fractions were pooled in two portions with different immune reactivity.

The data apply to the pooled fractions with approximately the highest immune reactivity.

cation procedures. Iso-electric focusing and chromatography on Sephadex LH-20 gave no fractions with increased immune reactivity. Table 1 shows the results of gel filtration on Sephadex G-75 and Biogel P-60, and of centrifugation on a linear 20–60% sucrose gradient at 283,000 g for 40 hr. Since centrifugation gave the products with the highest immune reactivity, the following experiments were performed with conjugates, purified by that method.

Fig. 2 and 3 show the displacement of E-HRP conjugates from their binding to antibodies by E<sub>1</sub>, E<sub>2</sub> and E<sub>3</sub>. Fig. 2 shows the curves obtained with an anti-E<sub>3</sub> serum combined with an E<sub>2</sub>-HRP and an E<sub>3</sub>-HRP, fig. 3 shows the curves for the same conjugates and an anti-E<sub>2</sub> serum. We obtained similar results with various other antisera, although no antiserum showed as large a difference in reaction with E<sub>2</sub>-HRP and E<sub>3</sub>-HRP as the one shown in fig. 3. With appropriate combinations of conjugate and antiserum, nanogram amounts of hapten could be detected.

In order to evaluate the specificity of the assay systems, we tested various compounds, including oestrogen sulphates and glucuronides and non-oestrogenic steroids, with two different conjugate-antiserum



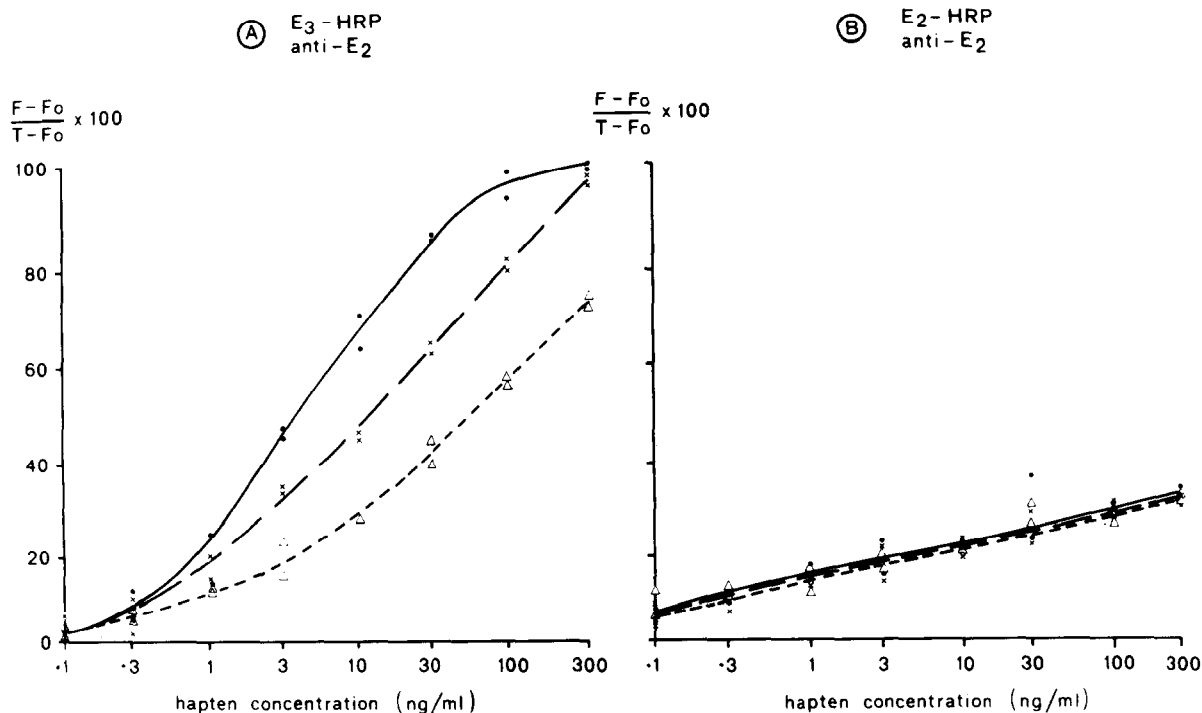


Fig. 3. Oestrogen assay systems: An E<sub>3</sub>-HRP (A) and an E<sub>2</sub>-HRP (B), with anti-E<sub>2</sub> serum 408 in dilutions of 1/700 and 1/1000, respectively. For explanation of the symbols see legend to fig. 2.

determined do not. The same effect may be expected in radioimmunoassays employing <sup>125</sup>I-labelled E-succinyl-BSA [13, 14], thus reducing the theoretical sensitivity gain from the higher specific activity.

Oestrogens conjugated with sulphate or glucuronide at site 3 do not react in our systems, whereas 16- or 17-glucuronide oestrogens do, in many cases even as well as the unconjugated oestrogen (table 2). This might be expected, since Midgley reported that structural differences at the end distal to conjugation have the greatest influence on the cross-reaction, while alterations near the site of conjugation have little effect [14]. Our E<sub>2</sub>-HRP/anti-E<sub>3</sub> system, which crossreacts completely with E<sub>3</sub>-16- and E<sub>3</sub>-17-glucuronide, is potentially applicable to E determination in pregnancy urine, where most of the E occurs as 16- or 17-conjugated E<sub>3</sub>. The application of a radioimmunoassay using anti-E<sub>3</sub> for E determination in pregnancy urine has been described [15].

## References

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Table 2

Cross-reaction<sup>†</sup> of various steroids in two oestrogen assay systems (means of two or three determinations).

| Hapten                         | E <sub>3</sub> -HRP<br>anti-E <sub>2</sub> 408 | E <sub>2</sub> -HRP<br>anti-E <sub>3</sub> 405 |
|--------------------------------|--|--|
| Oestrone                       | 100  | 100  |
| Oestrone-3-sulphate            | 0.3  | < 0.1  |
| Oestrone-3-glucuronide         | < 0.1  | < 0.1  |
| Oestradiol                     | 70   | 110  |
| Oestradiol-17-hemisuccinate    | 330 *  | 740  |
| Oestradiol-3-glucuronide       | 1  | 1  |
| Oestradiol-17-glucuronide      | 100  | 86   |
| Oestriol                       | 17   | 36   |
| Oestriol-16,17-dihemisuccinate | 22   | 1750 *   |
| Oestriol-3-sulphate            | < 0.1  | < 0.1  |
| Oestriol-3-glucuronide         | < 0.1  | < 0.1  |
| Oestriol-16-glucuronide        | 3 **   | 32 **  |
| Oestriol-17-glucuronide        | 7  | 49   |
| 16-Epi oestriol                | 20   | 55   |
| 17-Epi oestriol                | 8  | 27   |
| Testosterone                   | < 0.03   | < 0.03   |
| Progesterone                   | 0.1  | < 0.03   |
| Cortisone                      | < 0.03   | < 0.03   |
| Corticosterone                 | < 0.03   | < 0.03   |

<sup>†</sup> The cross-reaction is expressed as  

$$\frac{\text{amount of oestrone that displaces 50\% of enzyme activity}}{\text{amount of hapten that displaces 50\% of enzyme activity}} \times 100$$

\* Lack of parallelism with oestrone: steeper curve.

\*\* Lack of parallelism with oestrone: shallower curve.

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